

10/591798

IAP12 Rec'd PCT/PTO 06 SEP 2006

10009684WO01

1

DESCRIPTION

METHOD OF DETECTING NUCLEIC ACID

5 TECHNICAL FIELD

The present invention relates to a method of detecting a nucleic acid, and more particularly to a method of detecting a nucleic acid using a primer array and a PCR method.

10

BACKGROUND ART

Base sequence analysis of genome or the like of various living beings including human beings has intensively proceeded, with the result that gene analysis has come into use for diagnosis of a genetic disease, cancer, infectious disease, lifestyle-related disease, or the like; decision of a therapeutic strategy; check after treatment; and prognostic expectation. Moreover, in another field, gene analysis is beginning to be used for distribution management of foods such as meat and grain.

Among techniques used for the gene analysis, a nucleic acid array immobilized with plural nucleic acids such as DNA probes, oligonucleotide probes, and cDNA is one of the techniques that has particularly drawn attention since the late 1990s. The nucleic

acid array may also be referred to as DNA array, oligonucleotide array, cDNA array, or the like, and in some situations, as nucleic acid chip or DNA chip. Gene analysis techniques using a nucleic acid array or a nucleic acid array itself essentially have an advantage that multiple and various solid-phase hybridizations can be performed simultaneously, although which is not described in detail herein because those techniques have been generally well known recently.

On the other hand, the PCR (polymerase chain reaction) method that has been considered as an important technique for gene analysis does not go out of fashion today and is one technique most widely used in the gene analysis.

Since the PCR method was devised, some improvements and evolutions have been accomplished. For example, the traditional PCR method was performed for one gene or one base sequence in the form that a base sequence to be amplified was sandwiched by two primers. However, recently, PCR is performed using two primers sandwiching base sequences to be amplified that include different base sequences of plural genes or plural base sequences. That is, amplification of plural genes or the like can be realized using only two (common) primers. Such PCR method is generally referred to as a universal PCR

method. For example, when plural infecting organisms causing infectious diseases are to be identified, PCR is performed using primers which sandwich base sequences including a part of base sequences of a specific rRNA (for example, 16s rRNA) unique to the respective infecting organisms and have common base sequences in the respective infecting organisms, and the aforementioned unique base sequences are detected, to thereby identify and detect the respective infecting organisms. Such method has an advantage that PCR procedures are not required for the respective plural genes or the like.

On the other hand, a procedure called multiplex PCR has been developed. This procedure is intended to amplify plural genes or the like with different primer sets in one batch. Although this procedure has a difficulty in design, setting of a primer strand length, base sequence, and PCR conditions or the like, basically, plural and multiple PCR procedures can be performed simultaneously. Moreover, in some situations, improved quantitative detection can be expected through the reactions in the same batch.

Further, a procedure that is a combination of the above-described chip technique and PCR method has also been proposed.

There has been disclosed one procedure of so-

called solid phase PCR that is used for performing plural PCR procedures on one solid-phase by immobilizing a set of PCR primers including two oligonucleotides on a matrix in a nucleic acid array 5 (see Japanese Patent Application Laid-Open No. 2001-299346). According to such a procedure, two amplified products that have been amplified on the matrix form an inverted U-shaped hybridized product mutually by keeping the products under hybridization 10 conditions together with a nucleic acid array after PCR. A gene to be detected can be identified and detected by detecting the hybridized product using, for example, a fluorescent intercalator dye.

Moreover, a solid-phase PCR method that is 15 performed on a microplate having plural wells has been proposed (see THCH NOTE Vol. 3, No. 16, published by Nalge Nunc International). That is, the method of PCR in which, one primer of a set of PCR primers is covalently immobilized on the well surface 20 of the microplate, and a template nucleic acid and both primers are used in the wells for PCR. Detection is performed separately using a detection probe for PCR amplified products that are elongated from the primer immobilized on the well surfaces.

25 Conventional techniques relating to the present invention, that is, gene detection techniques, nucleic acid array techniques, PCR methods, and

solid-phase PCR methods have been outlined above.

The aforementioned solid-phase PCR is basically used for one PCR reaction on one matrix or one well. and it is difficult to be applied to the above-
5 described universal PCR or multiplex PCR. Moreover, in the method described in Japanese Patent Application Laid-Open No. 2001-299346, hybridization is sterically limited because an inverted U-shaped hybridized product is finally formed in a microregion.
10 Moreover, because the reactions of the aforementioned method described in TECH NOTE Vol. 3, No. 16 are performed in microplate wells, the numbers of the reactions are limited compared to reactions in the case of a nucleic acid array, and a large volume of a
15 reaction solution is required, so that the detection efficiency may need more improvement. Further, the detection in those techniques requires another step after amplification, which is performed using, for example, a fluorescent intercalator or another
20 detection probe.

Moreover, Japanese Patent Application Laid-Open No. 2003-523183 discloses an array for detecting and comparing expression patterns of plural target polynucleotides from at least two different
25 biological sources, which includes at least two groups of oligonucleotide primers immobilized on a discrete region of a solid-phase support. Each group

of oligonucleotide primers is selected for a specific target polynucleotide and includes a sequence complementary to the sequence of the specific target polynucleotide. In the array, each group can be
5 identified by the position on the solid-phase support. Further, the array can be used for detecting expression patterns of target polynucleotides from single biological source.

However, in order to detect different genes,
10 only one set of primers is prepared for each detection. In the case where different microorganisms each having extremely similar sequence, for example, infecting organisms of infectious diseases are identified, determination may not be
15 performed.

DISCLOSURE OF THE INVENTION

In view of the above-described circumstances, the present invention relates to a solid-phase PCR method which enables simple, high-efficiency, and high-precise detection of various genes, and an object of the present invention is to provide a method of detecting a nucleic acid which may be widely utilized in fields on the basis of gene
20 detection such as diagnosis, treatment, or prognostic expectation in the medical field; or distribution
25 management in the food field.

The aforementioned object is accomplished by the following aspects of the present invention.

More specifically, a first aspect of the present invention relates to a method of detecting a nucleic acid characterized by including the steps of:

(1) preparing a single-stranded nucleic acid having plural partial and sequential base sequences to be detected (A-strand) and a single-stranded nucleic acid having a base sequence complementary to a base sequence of the A-strand (B-strand);

(2) preparing nucleic acids as primers each having one of the plural base sequences to be detected, immobilizing the respective primers independently in separate regions on a substrate, and preparing a primer array in which the respective base sequences to be detected are distributed in the primer-immobilized regions;

(3) preparing a nucleic acid having a sequence complementary to a partial and sequential base sequence within the region between a 3'-end of the A-strand and the base sequence to be detected which is located nearest the 3'-end as a primer for elongating the B-strand;

(4) performing PCR reactions using the A-strand and B-strand as templates, and using the primers immobilized on the substrate, and the primer for elongating the B-strand;

- (5) forming a hybridized product of a nucleic acid corresponding to the A-strand which has been elongated and amplified as a result of the PCR reactions and bound to the substrate and a nucleic acid corresponding to the B-strand which has been elongated and amplified and has not bound to the substrate; and
- (6) detecting the base sequence to be detected by detecting the hybridized product in the respective primer-immobilized regions in the array.

The aforementioned method enables the universal PCR on a nucleic acid array.

A second aspect of the present invention relates to a method of detecting a nucleic acid characterized by including the steps of:

- (1) preparing plural single-stranded nucleic acids each having a partial and sequential base sequence to be detected (A-strand group: A₁-strand to A_n-strand: n ≥ 2) and a group of single-stranded nucleic acids each having a base sequence complementary to a base sequence of each strand of the A-strand group (B-strand group: B₁-strand to B_n-strand: n ≥ 2);
- (2) preparing nucleic acids as primers each having one of the plural base sequences to be detected, immobilizing the respective primers independently in separate regions on a substrate, and

preparing a primer array in which the respective base sequences to be detected are distributed in the primer-immobilized regions;

(3) preparing nucleic acids having a sequence
5 complementary to a partial and sequential base sequence within the region between a 3'-end of each strand of the A-strand group and the base sequence to be detected which is located nearest the 3'-end as primers for elongating the B-strands (PB-strand group: PB₁-strand to PB_n-strand n ≥ 2);

(4) performing PCR reactions using each strand of the A-strand group and each strand of B-strand group as templates, and using the primers immobilized on the substrate, and the plural primers for 10 elongating the B-strands of the PB-strand group;

(5) forming a hybridized product of a nucleic acid corresponding to the A-strand group which has been elongated and amplified as a result of the PCR reactions and bound to the substrate and a nucleic acid corresponding to the B-strand group which has 20 been elongated and amplified and has not bound to the substrate; and

(6) detecting the base sequence to be detected by detecting the hybridized product in the respective 25 primer-immobilized regions in the array.

In the aforementioned detection method, each A-strand may include one or more of the plural base

sequences to be detected.

The above-described methods enable the solid-phase universal PCR, solid-phase multiplex PCR and solid-phase universal multiplex PCR using the nucleic acid array, which have not been accomplished by conventional techniques.

Further, a third aspect of the present invention relates to a method of detecting a nucleic acid characterized by including the steps of:

10 (1) preparing a single-stranded nucleic acid having plural partial and sequential base sequences to be detected (A-strand) and a single-stranded nucleic acid having a base sequence complementary to a base sequence of the A-strand (B-strand);

15 (2) preparing nucleic acids as primers each having one of the plural base sequences to be detected, immobilizing the respective primers independently in separate regions on a substrate, and preparing a primer array in which the respective base sequences to be detected are distributed in the primer-immobilized regions;

20 (3) preparing a nucleic acid having a sequence complementary to a partial and sequential base sequence within the region between a 3'-end of the A-strand and the base sequence to be detected which is located nearest the 3'-end as a primer for elongating the B-strand;

(4) performing PCR reactions using the A-strand and the B-strand as templates, and using the primers immobilized on the substrate, and the primer for elongating the B-strand, and nucleotide monomers with 5 a part or all of at least one of the nucleotide monomers being labeled; and

10 (5) detecting a nucleic acid corresponding to the A-strand which has been elongated and amplified from a probe binding to the substrate via the label incorporated in the nucleic acid.

Further, a forth aspect of the present invention relates to a method of detecting a nucleic acid characterized by including the steps of:

15 (1) preparing plural single-stranded nucleic acids each having a partial and sequential base sequence to be detected (A-strand group: A₁-strand to A_n-strand: n ≥ 2) and a group of single-stranded nucleic acids each having a base sequence complementary to a base sequence of each strand of 20 the A-strand group (B-strand group: B₁-strand to B_n-strand: n ≥ 2);

25 (2) preparing nucleic acids as primers each having one of the plural base sequences to be detected, immobilizing the respective primers independently in separate regions on a substrate, and preparing a primer array in which the respective base sequences to be detected are distributed in the

primer-immobilized regions;

(3) preparing nucleic acids each having a sequence complementary to a partial and sequential base sequence within a region between a 3'-end of 5 each strand of the A-strand group and the base sequence to be detected which is located nearest the 3'-end as primers for elongating the B-strands (PB-strand group: PB₁-strand to PB_n-strand: n ≥ 2);

10 (4) performing PCR reactions using each strand of the A-strand group and each strand of the B-strand group as templates, and using the primers immobilized on the substrate and the plural primers for elongating the B-strands of the PB-stand group and nucleotide monomers with a part or all of at least 15 one of the nucleotide monomers being labeled; and

(5) detecting a nucleic acid corresponding to the A-strand which has been elongated and amplified from a probe binding to the substrate via the label incorporated in the nucleic acid.

20 In the aforementioned detection method, each A-strand may include one or more of the plural base sequences to be detected.

One feature of the third and forth aspects of 25 the present invention is to utilize a labeled monomer as a substitute for a part or all of at least one of nucleotide monomers used in PCR reactions in order to simplify a detection step. The method according to

the aspects enables the solid-phase PCR capable of performing detection immediately after PCR, solid-phase universal PCR capable of performing detection immediately after PCR, solid-phase multiplex PCR, or 5 solid-phase universal multiplex PCR without performing two-tiered steps in which hybridization is performed after PCR.

Further, the method according to the first to forth aspects of the present invention is 10 characterized in that a nucleic acid having the same base sequence as one selected from a region nearer the 5'-end of A-strand than a base sequence to be detected which is nearest the 5'-end (A-strand elongating primer) is added to the B-strand 15 elongating primer, and the nucleic acid is used as a primer which is not immobilized on a substrate, so that the amplifying efficiency of a target base sequence in PCR can be improved.

Moreover, in a further aspect of the method 20 according to the first to forth aspects of the present invention, the method is characterized in that PCR reactions and detection are performed in the form in which primer arrays are present in the same container. Further, the PCR reactions and detection 25 can be performed while being observed continuously using the same means.

Another aspect of the present invention relates

to a detection apparatus which enables the detection method of the present invention, characterized by including: at least a PCR reaction container; and detection means.

5 Moreover, the present invention includes provision of a kit for detecting a nucleic acid, including: the primer arrays; a PCR reaction reagent; and a nucleic acid detecting reagent.

10 Note that reference symbol "n" in the methods according to the respective aspects represents the same number (integer).

15 The present invention enables the solid-phase universal PCR, solid-phase multiplex PCR, or solid-phase universal multiplex PCR using nucleic acid arrays. Moreover, the present invention enables, by using a labeled nucleotide monomer in PCR, the solid-phase PCR capable of performing detection immediately after PCR, solid-phase universal PCR capable of performing detection immediately after PCR, solid-phase multiplex PCR, or solid-phase universal multiplex PCR.

20 That is, according to the present invention, simple, high-efficiency, and high-precise detection of various and multiple genes can be performed.

25 Other features and advantages of the present invention will be apparent from the following description taken in conjunction with the

accompanying drawings, in which like reference characters designate the same or similar parts throughout the figures thereof.

5 BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic view for illustrating the solid-phase universal PCR according to the present invention; and

10 Fig. 2 is a schematic view for illustrating the solid-phase universal PCR and incorporated label according to the present invention.

Fig. 3 schematically illustrates an apparatus for carrying out the method according to the invention.

15 Fig. 4 schematically illustrates another apparatus for carrying out the method according to the invention.

20 Fig. 5 schematically illustrates still another apparatus for carrying out the method according to the invention.

BEST MODE FOR CARRYING OUT THE INVENTION

Preferred embodiments of the present invention will now be described in detail in accordance with 25 the accompanying drawings.

A method of detecting a nucleic acid according to a first mode of the present invention includes the

aforementioned steps (1) to (6). This method enables the universal PCR on a primer array.

In this case, if the base sequence to be detected is, for example, of a gene having a double-stranded nucleic acid, the sense base sequence is basically the same as the anti-sense base sequence. It is obvious that analysis of the anti-sense sequence reveals the sense base sequence complementary to the sequence (that is, the base sequence of the gene).

Each operation in each step can be performed using the known method. In the detection step as the final step, any procedure which is capable of detecting a formed hybridized product may be used.

For example, a detection method using a fluorescent intercalator dye or fluorescent groove binder dye which interacts with a double-stranded nucleic acid to emit or enhance fluorescence may be utilized.

Alternatively, there may also be adopted a procedure for labeling a nucleic acid having a base sequence complementary to a base sequence located in the position nearer 3'-end than that of the 3'-end of the base sequence to be detected of A-strand, which is used as a primer in the step (4) (which is a B-strand elongating primer and a primer derived from a segment nearer the 3'-end of A-strand; herein, the phrase "derived from a position nearer the 3'-end" means

"derived from a segment located in the position nearer 3'-end than that of the base sequence to be detected which is nearest the 3'-end"), with a fluorescent dye such as fluorescein,
5 tetramethylrhodamine, Cy3, or Cy5, or radioisotope. Note that, in the case of using the fluorescent dye, a fluorescent microscope may be used for observation, and a step of observing a label using, for example, a confocal fluorescent microscope may further be
10 included.

In this method, one primer of a set of primers to be used PCR reactions is immobilized on a solid-phase, and the other (B-strand elongating primer) is dissolved in a reaction solution before use. The B-strand elongating primer has functions as a common primer for amplifying each base sequence to be detected. Further, the steps (2) and (3) of the first mode may be changed as follows:

(2) preparing as primers, nucleic acids each having one of the plural base sequences to be detected, immobilizing the respective primers independently in separate regions on a substrate, and preparing a primer array in which the respective base sequences to be detected are distributed in the primer-immobilized regions; and
25

(3) preparing a nucleic acid having a partial and sequential base sequence within the region

between a 5'-end of the A-strand and the base sequence to be detected which is located nearest the 5'-end as a primer for elongating the A-strand and preparing a nucleic acid having a base sequence 5 complementary to a partial and sequential base sequence within the region between a 3'-end of the A-strand and the base sequence to be detected which is located nearest the 3'-end as a primer for elongating the B-strand.

10 That is, in addition to the B-strand elongating primer, a nucleic acid having the same base sequence as a sequential and partial base sequence which is located in the position nearer 5'-end than that of the 5'-end of the base sequence to be detected of A-strand (which is an A-strand elongating primer and a primer derived from a segment nearer the 5'-end of A-strand: herein, the phrase "derived from a position nearer the 5'-end" means "derived from a segment located in the position nearer 5'-end than that of 15 the base sequence to be detected which is nearest the 5'-end") may be dissolved in a reaction solution to be used concomitantly as the second common primer. In some situations, the amplifying efficiency in such concomitant use may be higher.

20 Note that, in some situations, this method may include a step of removing a substance other than the aforementioned hybridized product (such as a template

nucleic acid, or a nucleotide monomer or enzyme used for PCR reactions) through a washing operation for removing a reaction solution on the substrate.

Those methods relate to the universal PCR as described above, and the method of detecting a nucleic acid according to a second mode of the present invention includes the above-described steps (1) to (6). This method enables the multiplex PCR.

In this case, the same detection method as that described above may be used for detecting a hybridized product. The aforementioned detection method may be used for plural single-stranded nucleic acids each having one or more partial and sequential base sequences to be detected (A-strand group: A₁-strand to A_n-strand: n ≥ 2). Moreover, the steps (2) and (3) according to the second mode may be changed as follows:

(2) preparing nucleic acids as primers each having one of the plural base sequences to be detected, immobilizing the respective primers independently in separate regions on a substrate, and preparing a primer array in which the respective base sequences to be detected are distributed in the primer-immobilized regions; and

(3) preparing nucleic acids each having a partial and sequential base sequence within the region between a 5'-end of each strand of the A-

strand group and the base sequence to be detected which is located nearest the 5'-end as primers for elongating the A-strand (PA-strand group: PA1-strand to PAn-strand: $n \geq 2$) and preparing nucleic acids
5 each having a base sequence complementary to a partial and sequential base sequence within the region between a 3'-end of each strand of the A-strand group and the base sequence to be detected which is located nearest the 3'-end as primers for
10 elongating the B-strand (PB-strand group: PB1-strand to PBn-strand: $n \geq 2$).
15

That is, in amplification reactions, plural nucleic acids each having the same base sequence as a sequential and partial base sequence which is located
15 in the position nearer 5'-end than that of the 5'-end of the base sequence to be detected of the aforementioned A-strand group (PA-strand group: PA1-strand to PAn-strand: $n \geq 2$) may be dissolved in a reaction solution together with the PB-strand group
20 to be used as common primers. In some situations, the amplification efficiency in this method is higher.
25

The aforementioned methods enable the solid-phase universal PCR, solid-phase multiplex PCR, or solid-phase universal multiplex PCR using the nucleic acid array.

Fig. 1 is a schematic view illustrating the aforementioned method in the case in which A-strand

has three base sequences to be detected. In Fig. 1,
a primer 1 to a primer 3 are located from the 3'-end
to the 5'-end of A-strand, and on B-strand which is
complementary to A-strand, a segment is defined as a
5 common primer (univ. anti-sense primer), which is
located on the segment having a sequence
complementary to that of the segment selected from
the region located in the position nearer 3'-end than
the 3'-end of the primer 1 which is nearest the 3'-
10 end of A-strand. Moreover, a segment is defined as a
common primer (univ. sense primer), which is located
on the segment having the same sequence as that in
the segment selected from the region located in the
position nearer 5'-end than the 5'-end of a primer 3
15 which is nearest the 5'-end of A-strand. The
substrate has regions in which the respective primers
having the same sequence as that of segments of the
primer 1 to the primer 3 are immobilized, regions in
which primers are immobilized are shown in Fig. 1.
20 When PCR reactions are performed on the substrate,
amplification and elongation proceed from the primers
immobilized on the substrate toward the location
predetermined by a common primer. In Fig. 1, at the
primer-immobilized region, the 3'-end of the primer
25 fixed at 5'-end on the substrate is elongated and
amplified to the position predetermined by a common
primer (univ. anti-sense primer) to form a fixed

strand corresponding to A-strand, and the fixed strand and a strand corresponding to B-strand which is amplified from B-strand may form a double-strand. By detecting the double-strand, a base sequence to be 5 detected in A-strand can be identified. When a common primer (univ. sense primer) is further added, the amplifying effect of A-strand itself may be expected, in some situations, the amplifying efficiency may be improved.

10 Next, as in the third and forth modes, in order to simplify detection steps, a labeled monomer may be utilized as a substitute for a nucleotide monomer used in PCR reactions. The situation is shown in a schematic view of Fig. 2. That is, when a labeling 15 substance is bound to a part or all of at least one of the nucleotide monomers for elongation which include bases of adenine, guanine, cytosine, and thiamine upon amplification, the aforementioned labeling substance is introduced to a nucleic acid 20 elongated from a primer immobilized on a substrate after amplification. As a result, even if steps subsequent to hybridization in the aforementioned method are omitted, detection can be performed. A labeling substance used in this method is not 25 particularly limited, but includes, for example, the above-described fluorescent dyes and radioisotopes. Also, in the case where a fluorescent dye is used for

labeling, in the detection steps in the third and forth modes, an observation step using a confocal fluorescent microscope may be additionally included.

Note that, in some situations, this method may
5 include a step of removing a substance other than a nucleic acid which is elongated and amplified by the PCR reactions and corresponds to A-strand binding to a substrate, for example, a nucleic acid which is elongated and amplified and corresponds to B-strand,
10 a template nucleic acid, a nucleotide monomer used in the PCR reactions, or an enzyme.

This method enables the solid-phase PCR capable of performing detection immediately after PCR, the solid-phase universal PCR capable of performing
15 detection immediately after PCR, the solid-phase multiplex PCR, or the solid-phase universal multiplex PCR without performing two-tiered steps in which hybridization is performed after PCR, and was required to be improved in the aforementioned
20 conventional technique.

(Other Embodiments)

An apparatus for realizing the detection methods described above fall within the scope of the present invention.

25 The above-described methods are preferred at least in points that PCR reactions and detection are performed in the form in which primer arrays are

present in the same container, which is preferred in
that an apparatus or the like can be simplified and
in that, as described below, PCR reactions and
detection can be performed using the same method
5 simultaneously and continuously. In such case, when
the PCR reactions and detection are performed using
the same method simultaneously and continuously, the
process of the PCR amplification can be monitored.
Accordingly, this is a preferred mode in that so-
10 called real-time PCR is realized, that is, highly
quantitative detection can be performed. An
apparatus that is equipped with a PCR reaction
container and detection method enabling such a
detection method is a preferred mode for carrying out
15 the present invention.

Fig. 3 illustrates a preferred embodiment of
such an apparatus. In the apparatus, the PCR
container comprises a substrate 1 having a surface 2
with immobilized polymers, a reaction chamber 3 and a
20 temperature controlling unit 4. The substrate is
transparent against wavelength used for detection.
The reaction chamber is facing to the surface. The
temperature controlling unit is placed at a position
not preventing operation of detection means 5 which
25 is placed on the side opposite to said surface in
relation to said substrate.

The solution mentioned above is introduced from

an inlet port (not shown) into the reaction chamber 3 to conduct PCR reactions while the temperature is controlled by the temperature controlling unit 4 in accordance with the aforementioned schedule.

5 Fig. 4 illustrates another embodiment of such an apparatus. In the apparatus of Fig. 4, the temperature controlling unit 4 is arranged such that it surrounds also the lateral surface of the reaction container to increase the contact area.

10 Fig. 5 illustrates still another embodiment of such an apparatus. In the apparatus of Fig. 5, the entire surface except the detection area is covered by the temperature controlling unit 4. Moreover, the present invention provides a kit for detecting a
15 nucleic acid, which includes the aforementioned primer arrays, PCR reaction reagent, and detection reagent. The above-described PCR reaction container may be provided for the kit, and the PCR reaction container may be formed into a cartridge form.

20 Moreover, in the case where a nucleic acid to be detected forms a double-stranded nucleic acid, the detection reagent of the above-described kit for detecting a nucleic acid is preferably a fluorescent dye as an intercalator or a groove binder, which acts
25 on a double-stranded nucleic acid as a fluorescent dye.

EXAMPLES

Hereinafter, the present invention will be described in detail with reference to examples.

Example 1 (Solid-phase universal PCR I)

(1) Preparation of primer

5 The following seven segments (Ec1 to Ec7) of sense base sequences of genome DNA of 16s ribosomal RNA (rRNA) of Escherischia coli (ATCC#11775) were defined as detection targets of a solid-phase universal PCR, which were selected as primers to be
10 immobilized on solid-phase.

Ec1 5' CTCTTGCATCGGATGTGCCCA 3' (SEQ ID NO: 1)
Ec2 5' ATACCTTGCTCATTGACGTTACCCG 3' (SEQ ID NO: 2)
Ec3 5' TTTGCTCATTGACGTTACCCGCAG 3' (SEQ ID NO: 3)
Ec4 5' ACTGGCAAGCTTGAGTCTCGTAGA 3' (SEQ ID NO: 4)
15 Ec5 5' ATACAAAGAGAAGCGACCTCGCG 3' (SEQ ID NO: 5)
Ec6 5' CGGACCTCATAAAGTGCCTCGTAGT 3' (SEQ ID NO: 6)
Ec7 5' GCGGGGAGGAAGGGAGTAAAGTTAAT 3' (SEQ ID NO: 7).

Those primers were synthesized by a synthesis company (BEX Co., Ltd.) on commission. The 5'-end of
20 each of Ec1 to Ec7 was bound to a thiol (SH) group via a linker for binding to a solid-phase substrate as described below. The Ec1 structure to which a thiol group is bound is shown below as an example.

HS(CH₂)₆OP(O₂)O-dCTCTTGCATCGGATGTGCCCA

25 Moreover, the following base sequence (EcAP) was selected as a base sequence of a common primer of the anti-sense sequence:

EcAP 5' ATCCAACCGCAGGTTCCCCTAC 3' (SEQ ID NO: 8)

EcAP was synthesized in a general manner. All the primers were deprotected and purified based on the conventional methods.

5 (2) Extraction of Escherischia coli genome DNA

Firstly, an E.coli standard strain was cultured in accordance with the conventional method. 1.0 ml of the microorganism-cultured medium ($OD_{600} = 0.7$) was collected in a 1.5-ml microtube, and the bacterial 10 cells were collected by centrifugation (8,500 rpm, 5 minutes, 4°C). After the supernatant was discarded, 300 μ l of Enzyme Buffer (50 mM Tris-HCl: p.H. 8.0, 25 mM EDTA) was added thereto, and the cells were resuspended using a mixer. The resuspended bacterial 15 suspension was centrifuged again, to thereby collect bacterial cells (8,500 rpm, 5 minutes, 4°C). After the supernatant was discarded, the following enzyme solutions were added to the collected bacterial cells, and the cells were resuspended using a mixer:

20 Lysozyme 50 μ l (20 mg/ml in Enzyme Buffer)
N-Acetylmuramidase SG 50 μ l (0.2 mg/ml in Enzyme Buffer).

Next, the resuspended bacterial suspension to which the enzyme solutions were added was left to 25 stand in an incubator at 37°C for 30 minutes, to thereby perform treatment for cell wall lysis.

Subsequently, genome DNA was extracted using a kit

for purifying a nucleic acid (Mag Extractor. Genome-: manufactured by TOYOBO Co., Ltd.).

Specifically, firstly, 750 µl of a dissolution/adsorption solution and 40 µl of magnetic beads were added to the pretreated microorganism suspension, and the mixture was stirred vigorously for 10 minutes using a tube mixer (Step 1).

The microtube was set on a separatory stand (Magical Trapper) and allowed to stand for 30 seconds, 10 to thereby collect the magnetic particles on the wall surface of the tube, and the supernatant was discarded while the tube was set on the stand (Step 2).

Next, 900 µl of a washing solution was added thereto, 15 and the particles were resuspended by stirring using the mixer for about 5 seconds (Step 3).

Next, the microtube was set on the separatory stand (Magical Trapper) and allowed to stand for 30 seconds, to thereby collect the magnetic particles on 20 the wall surface of the tube, and the supernatant was discarded while the tube was set on the stand (Step 4).

The procedures of Steps 3 and 4 were repeated, and the second washing was performed (Step 5). 25 Subsequently, 900 µl of 70% ethanol was added thereto, and the particles were resuspended by stirring using the mixer for about 5 seconds (Step 6).

Next, the microtube was set on the separatory stand (Magical Trapper) and allowed to stand for 30 seconds, to thereby collect the magnetic particles on the wall surface of the tube, and the supernatant was 5 discarded while the tube was set on the stand (Step 7).

The procedures of Steps 6 and 7 were repeated, and the second washing was performed with 70% ethanol 10 (Step 8). Subsequently, 100 µl of pure water was added to the collected magnetic particles, and the mixture was stirred for 10 minutes using the tube mixer.

Next, the microtube was set on the separatory stand (Magical Trapper) and allowed to stand for 30 seconds, to thereby collect the magnetic particles on 15 the wall surface of the tube, and the supernatant was collected in a new tube while the tube was set on the stand.

The collected genome DNA of Escherischia coli 20 was subjected to agarose electrophoresis and absorbance measurement with the wavelength of 260/280 nm in accordance with the conventional methods to assay its quality (amount of contaminated low-molecular-weight nucleic acids, the degree of degradation) and collected amount. In this example, 25 about 9 µg of the genome DNA was collected, and degradation of the genome DNA and contamination of

rRNA were not observed. The collected genome DNA was dissolved in TE buffer so as to have a final concentration of 50 ng/μl, and the mixture was used in the following examples.

5 (3) Manufacture of DNA array

(3-1) Washing of glass substrate

A glass substrate made of synthetic quartz (size: 25 mm × 75 mm × 1 mm, manufactured by Iiyama Tokusyu Glass Co. Ltd.) was placed in a heat-resistant and alkali-resistant rack and immersed in a washing solution for ultrasonic cleaning which had been adjusted to a predetermined concentration. The substrate was immersed in the washing solution overnight, and ultrasonic cleaning was then performed for 20 minutes. Subsequently, the substrate was taken out and lightly rinsed with pure water, followed by ultrasonic cleaning in ultrapure water for 20 minutes. Next, the substrate was immersed in a 1N sodium hydroxide solution heated to 80°C for 10 minutes. Washing with pure water and washing with ultrapure water were performed again, to thereby prepare a quartz glass substrate for a DNA array.

(3-2) Surface treatment

A silane coupling agent KBM-603 (manufactured by Shin-Etsu Silicones) was dissolved in pure water so as to have a concentration of 1%, and the mixture was stirred for two hours at room temperature.

Subsequently, the glass substrate that had been previously washed was immersed in the aqueous solution of the silane coupling agent and allowed to stand for 20 minutes at room temperature. The glass 5 substrate was drawn up, and its surface was lightly washed with pure water. Subsequently, nitrogen gas was blown on both the sides of the substrate, and the substrate was dried. Next, the treatment with the coupling agent was completed by baking the dried 10 substrate in an oven heated to 120°C for 1 hour, to thereby introduce amino groups to the substrate surface. Next, N-(6-maleimidocaproyloxy)succinimido (hereinafter, abbreviated as EMCS; produced by Dojindo Laboratories) was dissolved in a mixed 15 solvent of dimethylsulfoxide and ethanol (1:1) so as to have a final concentration of 0.3 mg/ml to prepare an EMCS solution. The baked glass substrate was left to cool and immersed in the prepared EMCS solution at room temperature for 2 hours. Through such treatment, 20 the amino groups introduced onto the surface by using the silane coupling agent reacted with succinimido groups of EMCS, to thereby introduce maleimide groups onto the surface of the glass substrate. The glass substrate was drawn up from the EMCS solution, and 25 the substrate was washed with the above-described mixed solvent and further washed with ethanol, followed by drying under nitrogen gas atmosphere.

(3-3) Primers

The primers to be immobilized on solid-phase (Ec1 to Ec7) described in (1) were separately dissolved in pure water, and each mixture was 5 dispensed so as to have a final concentration (when it was dissolved as described below) of 10 µM, followed by freeze-drying, to thereby remove water.

(3-4) Discharge of primer DNA by BJ printer, and binding to substrate

10 An aqueous solution (mixed solvent) containing 7.5 wt% of glycerin, 7.5 wt% of thiodiglycol, 7.5 wt% of urea, and 1.0 wt% of Acetylenol EH (manufactured by Kawaken Fine Chemicals Co., Ltd.) was prepared. Subsequently, each of previously prepared 7 primers 15 was dissolved in the aforementioned mixed solvent so as to have a prescribed concentration. The resultant DNA solution was filled into an ink tank for a bubble jet printer (trade name: BJF-850, manufactured by Canon Inc.), and the tank was attached to a print 20 head.

Note that the bubble jet printer used herein had been modified so as to enable print on a flat plate. Moreover, the bubble jet printer can perform spotting of about 5 pl of a DNA solution at a pitch 25 of about 120 µm by inputting a printing pattern in accordance with a predetermined file creation method.

Subsequently, a printing operation for one

glass substrate was performed using the modified bubble jet printer for preparing a DNA array. After confirming that printing was performed without fail, the array was allowed to stand in a humidified 5 chamber for 30 minutes to react maleimide groups on the surface of the glass substrate with thiol groups at the end of the nucleic acid primer.

(3-5) Washing

After the reaction was performed for 30 minutes, 10 the DNA solution remaining on the surface was washed out with 10 mM of phosphate buffer (pH 7.0) containing 100 mM of NaCl, to thereby yield a DNA array in which single-stranded DNA was immobilized on the surface of the glass substrate.

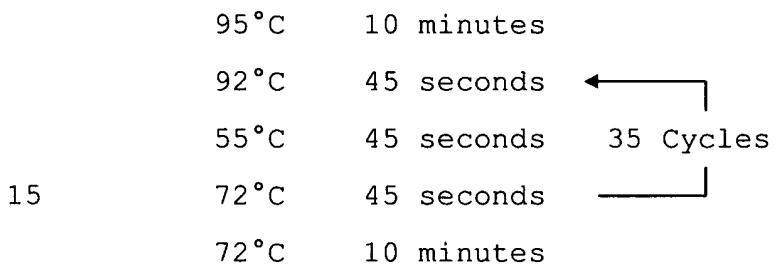
15 (4) Solid-phase PCR

A method of amplifying Escherischia coli genome DNA was described below.

Premix PCR reagent (TAKARA ExTaq)	25 µl
Template Escherischia coli genome DNA	2 µl (100 ng)
Primer EcAP	2 µl (20 pmole)
H ₂ O	21 µl
Total	50 µl

25 A microchamber in which a primer-binding site of the above-described primer array was covered and the temperature can be controlled was manufactured,

and a reaction solution of the aforementioned composition was sealed in the chamber. Then, amplification reactions were performed in accordance with the following protocol. More specifically,
5 after an incubation step at 95°C/10 minutes, a denaturation step at 92°C/45 seconds, an annealing step at 55°C/45 seconds, and an elongation step at 72°C/45 seconds were defined as one cycle, and the cycle was repeated 35 times, and finally an
10 incubation step at 72°C/10 minutes was performed.



After the completion of the reactions, 1 µl of a solution prepared by previously diluting a dye of which (fluorescence is enhanced under coexistence of a double-stranded nucleic acid, SYBR (registered trademark) Green I (Molecular Probes: trade name SYBR (registered trademark) Green I nucleic acid gel stain 10,000 × concentrate in DMSO) to 200-fold with pure
20 water was added to the chamber, and the primer array was placed under the conditions of 65°C/3 minutes → 92°C/2 minutes→ 45°C/3 hours for hybridization.
25

Next, the primer array was washed under the conditions of 2 × SSC/0.1% SDS (25°C)/2 minutes → 2 × SSC (20°C)/2 minutes → pure water (10°C)/2 minutes, and the array was taken out of the chamber and dried.

5 (5) Fluorescence measurement

The DNA array after the completion of the hybridization reaction was subjected to fluorescence measurement using a fluorescence detecting apparatus for DNA array (manufactured by Axon Instruments, 10 GenePix 4000B) (excitation wavelength: 532 nm, photomultiplier voltage: 400 V). The measurement results are shown in Table 1. Note that, in this example, all the operations are performed twice, so that the results were separately shown in Table 1.

15

Table 1

Primer No.	Fluorescence luminance	
	First time	Second time
Ec1	8,700	8,500
Ec2	9,100	9,050
Ec3	7,750	7,650
Ec4	11,000	11,000
Ec5	10,400	10,550
Ec6	7,400	7,400
Ec7	5,900	6,000

The numeric values of the fluorescent luminance in Table 1 represent pixel average luminance

(resolution: 5 μm). As is clear from Table 1, the results obtained by amplifying genome DNA extracted from Escherischia coli by the solid-phase universal PCR can be detected with high reproducibility.

5 Example 2 (Solid-phase universal PCR II)

The solid-phase PCR and fluorescence detection were performed in accordance with the same method as that in Example 1 except that, in addition to the primers used for the solid-phase PCR in Example 1, a 10 common primer (EcSP) of the sense sequence having the following base sequence was used at the same concentration as that of a common primer of the anti-sense sequence.

EcSP 5' GCGGCAGGCCTAACACATGCAAG 3' (SEQ ID NO: 9).

15 In Example 2, when the solid-phase PCR cycle was repeated 31 times, substantially the same results as those in Example 1 were obtained. The results reveal that the efficiency of the solid-phase PCR could be improved by letting both the common primers 20 of the sense sequence and anti-sense sequence coexist in a solution.

Example 3 (Solid-phase multiplex universal PCR I)

(1) Preparation of primers

In this example, genome DNA of rRNA of 25 Pseudomonas aeruginosa (ATCC#10145) will be detected simultaneously with Escherischia coli detected in Example 1. For this purpose, firstly, 8 thiolized

primers of the sense sequence for *Pseudomonas aeruginosa* (Pa1 to Pa8) were synthesized in the same way as that in Example 1. The base sequences of the primers are shown below.

5 Pa1 5' TGAGGGAGAAAGTGGGGATCTTC 3' (SEQ ID NO: 10)
Pa2 5' TCAGATGAGCCTAGGTGGATTAGC 3' (SEQ ID NO: 11)
Pa3 5' GAGCTAGAGTACGGTAGAGGGTGG 3' (SEQ ID NO: 12)
Pa4 5' GTACGGTAGAGGGTGGTGGATTTC 3' (SEQ ID NO: 13)
Pa5 5' GACCACCTGGACTGATACTGACAC 3' (SEQ ID NO: 14)
10 Pa6 5' TGGCCTTGACATGCTGAGAACCTTC 3' (SEQ ID NO: 15)
Pa7 5' TTAGTTACCAGCACCTCGGGTGG 3' (SEQ ID NO: 16)
Pa8 5' TAGTCTAACCGCAAGGGGGACG 3' (SEQ ID NO: 17).

Moreover, the following base sequence (PaAP) was selected as a base sequence of a common primer of the anti-sense sequence:

15 PaAP 5' ATCCAGCCGCAGGTTCCCCTAC 3' (SEQ ID NO: 18).

(2) Fluorescence detection

Extraction of *Pseudomonas aeruginosa* genome, manufacture of a DNA microarray immobilized with Escherischia coli primers and *Pseudomonas aeruginosa* primers, solid-phase PCR (Escherischia coli genome DNA and *Pseudomonas aeruginosa* genome DNA: 10 ng each, EcAP and PaAP: 20 pmole each), hybridization, washing, etc. were performed in the same way as that in Example 1, and fluorescence detection was performed. The results are shown in Table 2.

Table 2

Primer No.	Fluorescence luminance	
	Escherischia coli	Pseudomonas aeruginosa
Pa1	5,800	300
Pa2	6,050	550
Pa3	5,150	1,700
Pa4	7,400	650
Pa5	6,900	1,600
Pa6	4,900	2,650
Pa7	3,900	600
Pa8	-	350

Table 2 shows that all the given primer sequences of rRNA genomes of both Escherischia coli and Pseudomonas aeruginosa were simultaneously detected by the solid-phase multiplex universal PCR.
 5 Example 4 (Solid-phase multiplex universal PCR II)

The solid-phase PCR and fluorescence detection were performed in the same manner as that in Example 10 3 except that, in addition to the primers used for the solid-phase PCR in Example 2, common primers of the respective sense sequences of Escherischia coli and Pseudomonas aeruginosa (EcSP and PaSP) were used at the same concentration. The base sequence of PaSP 15 is shown below.

PaSP 5' GCGGCAGGCTTAACACATGCAAG 3' (SEQ ID NO: 19).

In Example 4, under the condition that the

cycle of the solid-phase PCR was repeated at given times fewer than those in Example 3 by about one or more, almost the same results as those in Example 3 were obtained. The results reveal that, in the 5 solid-phase multiplex universal PCR, the efficiency of the solid-phase PCR could be improved by letting both the common primers of the sense sequence and anti-sense sequence coexist in a solution.

Example 5 (Incorporated-label solid-phase universal 10 PCR)

Extraction of Escherischia coli genome, synthesis of primers, and manufacture of a primer array were performed in completely the same manner as that in Example 1, and the solid-phase PCR was then 15 performed using a PCR solution of the following composition.

Premix PCR reagent (TAKARA ExTaq)	25 µl
Template Escherischia coli genome DNA	2 µl (100 ng)
Primer EcAP	2 µl (20 pmole)
Cy-3 dUTP (Amersham Biosciences K.K.: 1 mM)	2 µl (2 nmol)
H ₂ O	19 µl
Total	50 µl

25

Subsequently, the primer array was washed, without keeping the same under to hybridization

conditions, under the conditions of 2 × SSC/0.1% SDS (92°C)/2 minutes → 2 × SSC/0.1% SDS (92°C)/2 minutes → 2 × SSC/0.1% SDS (25°C)/2 minutes → 2 × SSC (20°C)/2 minutes → pure water (20°C)/2 minutes, and
 5 the array was taken out of the chamber and dried.

Subsequently, fluorescence detection was performed in the same manner as that in Example 1. The results are shown in Table 3.

10

Table 3

Primer No.	Escherischia coli
	Fluorescence luminance
Ec1	11,500
Ec2	11,900
Ec3	10,000
Ec4	14,800
Ec5	13,200
Ec6	9,500
Ec7	7,700

15

In accordance with the detection method of the present invention, fluorescence detection was performed adopting an incorporated label in the solid-phase universal PCR. As a result, fluorescence detection was easily accomplished as shown in Table 3. Accordingly, the results reveal that fluorescence detection can be performed without requiring the hybridization step.

Example 6

The solid-phase universal PCR or solid-phase multiplex universal PCR in Examples 2 to 4 was performed using the incorporated label in Example 5.

- 5 Although the respective fluorescence intensities were different from those in Examples 2 to 4, the results reveal that the rRNA genome DNA of Escherichia coli or the rRNA genome DNAs of both Escherichia coli and Pseudomonas aeruginosa can be detected simultaneously.
- 10 Example 7 (Common primer-labeled solid-phase universal PCR)

Detection of Escherichia coli genome DNA was performed by the solid-phase universal method in completely the same manner as that in Example 1 except that the common primer EcAP was labeled with tetramethylrhodamine, hybridization was performed without coexistence with a fluorescent dye such as SYBR green, and fluorescence detection was performed using the aforementioned tetramethylrhodamine. The 15 structure of tetramethylrhodamine-labeled EcAP is shown below.

20

5' Rho-CONH(CH₂)₆OP(O₂)O-d ATCCAACCGCAGGTTCCCCTAC 3'
(Rho = tetramethylrhodamine).

As a result, almost the same results as those 25 in Example 1 were obtained although the differences in fluorescence intensities were observed.

A method of labeling the common primer of anti-

sense sequence was performed in the methods of Examples 2 to 4. As a result, the results confirm that all the methods were effective were obtained.

The present invention is not limited to the above embodiments and various changes and modifications can be made within the spirit and scope of the present invention. Therefore to apprise the public of the scope of the present invention, the following claims are made.

10

This application claims priority from Japanese Patent Application No. 2004-070986 filed on March 12, 2004, which is hereby incorporated by reference
15 herein.